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Association of ADHD with Genetic Variants in the 5'-Region of the Dopamine Transporter Gene: Evidence for Allelic Heterogeneity

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Multiple studies have reported an association between attention deficit hyperactivity disorder (ADHD) and the 10-repeat allele of a variable number tandem repeat (VNTR) polymorphism in the 3'-untranslated region (3'UTR) of the dopamine transporter gene (*DAT1*). Yet, recent meta-analyses of available data find little or no evidence for this association; although there is strong evidence for heterogeneity between datasets. This pattern of findings could arise for several reasons including the presence of relatively rare risk alleles on common haplotype backgrounds or the functional interaction of two or more loci within the gene. We previously described the importance of a specific haplotype at the 3' end of *DAT1*, as well as the identification of associated single nucleotide polymorphisms (SNPs) within or close to 5' regulatory sequences. In this study we replicate the association of SNPs at the 5' end of the gene and identify a specific risk haplotype spanning the 5' and 3' markers. These findings indicate the presence of at least two loci associated with ADHD within the *DAT1* gene and suggest that either additive or interaction effects of these two loci on the risk for ADHD. Overall these data provide further evidence that

genetic variants of the dopamine transporter gene confer an increased risk for ADHD.

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KEY WORDS: attention deficit hyperactivity disorder (ADHD); dopamine transporter gene (*DAT1*); association study

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INTRODUCTION

Attention deficit hyperactivity disorder (ADHD) is one of the most common and highly heritable behavioral disorders in children. The disorder is characterized by the childhood onset of age inappropriate and impairing levels of hyperactivity, impulsivity and inattention (American Psychiatric Association, 2000) that persists into adult life in around 65% of cases, either as the full-condition or in partial remission with persistence of symptoms associated with significant clinical impairments [Faraone et al., 2006]. The risk to siblings of ADHD probands is around four- to fivefold the population risk and heritability is estimated to be around 76% [Faraone et al., 2005]. The search for genetic variants that increase the risk for ADHD has focused mainly on the neurotransmitter systems involved in the response of ADHD symptoms to dopaminergic and noradrenergic medications. Genetic associations have been clearly demonstrated between ADHD and a variable number tandem repeat (VNTR) polymorphism in the dopamine D4 receptor gene and a simple sequence repeat polymorphism that lies upstream to the dopamine D5 receptor gene [Li et al., 2006]. Several other genes involved in the

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regulation of catecholamine neurotransmission are associated with ADHD in three or more studies and show small yet significant evidence of association [Asherson, 2004; Faraone et al., 2005].

One of the most interesting findings replicated in several studies is the association between the 10-repeat allele of a VNTR located within the 3' untranslated (3'UTR) region of the dopamine transporter gene (*DAT1*). The dopamine transporter is the main site of action of stimulant medications, which provide a marked and rapid reduction in the level of ADHD symptoms. Yet a recent study found no overall effect in a comprehensive meta-analysis of available association data [Li et al., 2006]. Despite this, the association remains of considerable interest due to the number of positive reports and the presence of significant evidence of heterogeneity across the datasets, indicating that a subset of datasets may show true association [Li et al., 2006]. One potential cause of heterogeneity could occur if only a subset of individuals carrying the 10-repeat allele is at risk for ADHD. This could arise if the *DAT1* risk allele interacts with additional genetic or environmental risk factors, which vary in frequency in different populations. For example data from several authors have indicated potential interactions with prenatal environmental risks [Kahn et al., 2003; Brookes et al., 2006a; Neuman et al., 2007].

An alternative explanation, supported by recent data, is that the 10-repeat allele is not the causative allele itself, but rather 'tags' a nearby functional variant that is only partially correlated with the 10-repeat allele, or that the 10-repeat allele interacts with a second locus within the *DAT1* gene [Asherson et al., 2007]. Evidence for this comes from the observation in several studies that specific *DAT1* haplotypes containing the 10-repeat allele confer risk for ADHD [Barr et al., 2001; Greenwood et al., 2002; Hawi et al., 2003; Galili-Weissstut et al., 2005; Brookes et al., 2006a]. A key finding from our own research was the observation in two independent samples from the UK and Taiwan of association between ADHD and a specific haplotype of the 10-repeat allele with the 6-repeat allele of a VNTR located in intron 8 [Brookes et al., 2006a]. This finding was subsequently replicated in the first set of samples (ST1) from the International Multi-centre ADHD Genetics (IMAGE) project, using a large sample of ADHD combined subtype cases [Asherson et al., 2007].

In an earlier study, the IMAGE ST1 samples were also screened for association with 32 SNPs and two VNTRs spanning *DAT1*, as part of a study that included the analysis of 51 candidate genes [Brookes et al., 2006b]. None of the markers were significant when adjusted for the number of markers investigated, but we reported significant gene-wide association for the sum of chi-squares for SNP associations across the gene. The analysis of two additional VNTR markers located within introns 3 and 14 was subsequently completed. To summarize the data from ST1, we found six SNP markers and three VNTR markers showing nominal evidence of association with combined type ADHD ($P \leq 0.05$,

Table I). The association signal fell into two groups of markers at the 3' and 5' ends of the gene. Genetic variants within each region were found to be in high linkage disequilibrium (LD), but there was low LD between the two regions, suggesting the presence of two independent loci associated with ADHD. In this study we extend these findings by investigating the association of four SNP markers in the 3' and 5' region (two from each region) that were associated with ADHD in the ST1 sample, in a second set of samples (ST2) collected by the IMAGE consortium. We further analyze the entire dataset and look for evidence of additive effects between the two ends of the gene.

METHODS

The IMAGE Sample

European Caucasian subjects were recruited from twelve specialist clinics in eight countries: Belgium, Germany, Holland, Ireland, Israel, Spain, Switzerland, and United Kingdom. Ethical approval for the study was obtained from National Institute of Health registered ethical review boards for each centre. Detailed information sheets were provided and informed consent obtained from the majority of children and from all of their parents. All ADHD probands and their siblings were aged 5–17 at the time of entry into the study and access was required to one or both biological parents for DNA collection. Entry criteria for probands were a clinical diagnosis of DSM-IV combined subtype ADHD and having one or more full siblings available for ascertainment of clinical information and DNA collection. Exclusion criteria applying to both probands and siblings include autism, epilepsy, IQ < 70, brain disorders and any genetic or medical disorder associated with externalizing behaviors that might mimic ADHD. Inclusion criteria included white European ancestry and living at home with at least one biological parent.

The diagnosis of ADHD was made following a parent interview with the Parental Account of Child Symptoms interview [PACS; Taylor et al., 1986] that asks about ADHD symptoms in various settings. An algorithm was used to derive each of the DSM-IV ADHD symptoms from the PACS interview data and these were combined with items that scored two or more from teacher ratings of DSM-IV items taken from the long version of the Conners' Teacher Rating Scale [Conners, 1995]. The diagnosis of ADHD was made if sufficient items were identified to fulfill DSM-IV criteria, and both impairment (based on severity of symptoms identified in the PACS interview) and pervasiveness (based on the presence of ADHD symptoms in more than one setting from PACS and scoring more than one item on the teacher Conners) were present. In 28 cases where no Conners data was present pervasiveness was defined on the basis of PACS data alone [Brookes et al., 2006b]. Further details on the clinical evaluation of the ADHD probands can be found in the original study of 51 candidate genes in the ST1 sample [Brookes et al., 2006b].

TABLE I. Markers Within the IMAGE Stage 1 Dataset (ST1) Displaying Nominal Significance ($P \leq 0.056$) for Association With ADHD

Marker	Position (bp)	Location	MAF	P value	Allele	T	NT	OR
3'UTR VNTR	—	3'UTR	0.29	0.03	10-repeat	277	245	1.13
Intron 14 VNTR	—	Intron 14	0.07	0.06	Insertion	106	80	1.33
rs40184	1,448,077	Intron 13	0.44	0.005	G	349	278	1.26
rs3776513	1,460,104	Intron 10	0.18	0.05	C	183	147	1.24
Intron 8 VNTR	—	Intron 8	0.22	0.006	6-repeat	243	207	1.17
rs2652511	1,499,389	5' Flanking	0.41	0.02	G	353	293	1.20
rs11564750	1,500,762	5' Flanking	0.09	0.03	G	127	94	1.35
rs10070282	1,503,444	5' Flanking	0.42	0.01	A	358	294	1.22
rs2550946	1,503,513	5' Flanking	0.42	0.007	G	358	289	1.24

Stage 2 (ST2) Sample and Combined ST1 + ST2 Sample

We previously reported *DAT1* genotype data from the analysis of the ST1 sample, which consisted of 776 DSM-IV combined type ADHD cases with DNA from both parents available for 90% of families and from one parent in 7% of families [Brookes et al., 2006b]. In this study we report on new data from the ST2 sample consisting of a further 435 combined type probands (376 probands plus 59 affected siblings). DNA was available from both parents in 80% of the families and from one parent in 19.2% of the families. Following the accumulation of further clinical information and a comprehensive audit of the entire clinical and DNA datasets completed in April 2007, the final set of individuals with combined type ADHD and fulfilling all inclusion and exclusion criteria in ST1 and ST2 were amended: the final combined ST1 and ST2 dataset used in this study consisted of 1,147 individuals with DSM-IV combined subtype ADHD from 988 affected probands and 150 of their affected siblings.

Genotyping and SNP Selection

The initial scan of ST1 samples identified two clusters of associated SNP markers at the 3' and 5' ends of the gene [Brookes et al., 2006b]. From these we selected two SNPs from the 3' region (rs40184 and rs3776513) and two located in the 5' region (rs2550946 and rs11564750). Since three of the associated 5' SNPs in the ST1 study were found to be in strong LD with an average r^2 of 0.97 (rs2622511, rs2550946, rs10070282) we selected only one (rs2550946) for analysis in ST2, whereas the other marker we selected (rs11564750) was only weakly associated with this group of SNPs (average r^2 of 0.14). Genotyping of the 3'UTR and intron 8 VNTRs were previously carried out in the ST2 sample and the results reported elsewhere [Asherson et al., 2007].

The SNP markers were genotyped using the ABI SNPLex [Tobler et al., 2005] and ABI TaqMan genotyping protocols (Applied Biosystems, Foster City, CA). The selected markers were initially analyzed on the ABI SNPLex system. Due to a high failure rate with the SNPLex assays, genotypes were repeated using the ABI TaqMan assay. We found no discrepancies in overlapping genotypes using the two methods. Standard protocols provided by ABI were followed for both types of assays. Three SNP assays were designed for the TaqMan 7900HT: SNP rs40184 was available from 'assays-on-demand', whilst SNP markers rs11564750 and rs2550946 were custom designed for the project. We were unable to design a TaqMan assay for SNP rs3776513.

Analysis

The transmission disequilibrium test was performed for probands with the combined subtype of ADHD using the

UNPHASED program (TDTPhase; [Dudbridge, 2003, www.rfcgr.mrc.ac.uk/~fdudbrid/software/unphased]). Nominal evidence for association was determined by *P*-values less than 0.05 and adjusted significance estimated by permutation tests. In addition we utilized the WHAP program to conduct conditional analyses that test the contribution of genetic variants to haplotype associations (www.genome.wi.mit.edu/~shaun/whap). In the conditional test a SNP is dropped from the null model and compared to the alternative model. A significant *P*-value indicates that the dropped SNP has an independent effect and makes a significant contribution to the haplotype association.

RESULTS

LD Across the Gene

Using the data from the ST1 study, we examined the LD structure across the gene, with values provided from the UNPHASED software package [Genro et al., 2007] (Fig. S1). Observation of D' values indicate that there are four main LD blocks: one of the LD regions spans the 3'UTR, another spans intron 11 to intron 8, a third spans exon 5 to intron 2 (rs403636) and a fourth spans the 5'UTR and upstream flanking region. These data indicate that the two regions of ADHD-associated markers identified at the 3' and 5' ends of the gene in the previous study represent independent SNP clusters and cannot be explained by one set of SNPs tagging the other.

Replication Study

Genotype data from the four SNPs in the ST2 dataset showed similar minor allele frequencies to those observed in the ST1 study (Table II). The four SNPs were in Hardy-Weinberg equilibrium and had a genotype success rate of over 97%; except for rs3776513, which could not be genotyped on the TaqMan platform and only 77% of the ST2 genotypes could be reliably called from the SNPLex assay. TDT analysis found that two of the four SNP markers showed nominal significance ($P < 0.01$) and surpassed the Bonferroni correction for the four markers tested: rs11564750 located in the 5' promoter region and rs3776513 located within intron 10 (Table II). For both these SNP markers the same allele was preferentially transmitted in both the ST1 and ST2 datasets. The remaining two SNP markers rs2550946 (5'-region), and rs40184 (intron 13) were not significantly associated, however both SNP markers showed over-transmission of the same allele observed to be the risk allele in the ST1 dataset. In the combined analysis of the ST1 and ST2 datasets, nominally significant associations were observed for all four SNP markers with estimated odds ratios in the range 1.2–1.5.

TABLE II. Findings From TDT Analysis of the ST1, and ST2 Datasets Independently and When Combined

SNP ID	Position (bp)	Location	Dataset	Minor allele	MAF	TDT P value	Risk allele	T	NT	OR
rs2550946	1,503,513	5' Promoter	ST1	A	0.42	0.006	G	358	289	1.24
			ST2	A	0.41	0.45	G	131	119	1.1
			ST1 and ST2	A	0.42	0.01	G	485	408	1.19
rs11564750	1,500,762	5' Promoter	ST1	C	0.09	0.03	G	127	94	1.35
			ST2	C	0.1	0.004	G	67	38	1.76
			ST1 and ST2	C	0.09	0.0006	G	195	133	1.47
rs3776513	1,460,104	Intron 10	ST1	T	0.18	0.05	G	183	147	1.24
			ST2	T	0.21	0.01	G	80	51	1.57
			ST1 and ST2	T	0.19	0.009	G	247	193	1.28
rs40184	1,448,077	Intron 13	ST1	T	0.44	0.005	C	349	278	1.26
			ST2	T	0.46	0.31	C	149	132	1.13
			ST1 and ST2	T	0.45	0.005	C	498	414	1.2

MAF, minor allele frequency.

Haplotype Analysis and Conditional Tests

Haplotype analysis was performed for the four markers genotyped in both the ST1 and ST2 datasets (Table III). Pair-wise haplotype analysis indicated that the most significant finding occurred between rs2550946 in the 5' promoter region and rs40184 in intron 13 ($P < 0.00002$). These two markers lie over 55 kb apart and have an estimated r^2 of 0.02 for transmitted alleles and an r^2 of 0.0002 for non-transmitted alleles. The four-marker haplotype has a global P -value of 0.0001 with only one haplotype (GGGC) displaying significant over transmission to ADHD probands (haplotype-specific P -value = 1.8×10^{-6}); these alleles coincide with those transmitted in the single marker analysis (Table IV).

WHAP analysis confirmed the haplotype association, but with reduced significance using the permutation routine ($P = 0.004$). When testing the effect of dropping each marker from the two marker haplotypes, a significant difference of fit was observed for both rs40184 ($P = 0.008$) and rs2550946 ($P = 0.03$). These findings indicate that independent markers at the 3' and 5' ends of the genes both contribute significantly to the haplotype association. In contrast, when the two 3' SNP marker and the two 5' SNP marker haplotypes were examined it was found that one of the two markers from each group could be dropped from the analysis, indicating that no additional information was obtained from the analysis of haplotypes within the 3' and within the 5' SNP clusters.

DISCUSSION

There are two main novel findings in this study. First, we replicate earlier evidence of association with between ADHD and SNP markers that span genetic variation in the 5' regulatory region of *DAT1*, including the promoter region. Second we show that markers that form independent SNP clusters at the 3' and 5' ends of the gene contribute significantly to a specific haplotype association. This is a key finding that suggests two independent functional sites within the *DAT1* gene associated with ADHD.

The ST2 IMAGE samples used in this study provide a small but clinically homogenous sample that was ascertained using identical clinical procedures to the ST1 sample. For this reason replication is feasible despite the relatively small sample size of ST2 compared to ST1. The selection of the four SNP markers was based on nominally significant associations observed in the ST1 study [Brookes et al., 2006b] with two SNP markers selected from each of two associated SNP clusters. Significant association with ADHD was observed for two of the markers rs11564750 and rs3776513 located in the 5' promoter region and intron 10, respectively, which is a direct replication of findings from ST1. Although the other two SNPs were not significantly associated with ADHD in ST2, they showed the same direction of effect for over-transmission of alleles to affected offspring. Analysis of the two SNPs that were not associated with ADHD in ST2 might also lack power, because

TABLE III. Significance Values for Two-Marker Haplotypes for the SNP Markers Investigated in the Combined ST1 and ST2 Dataset Are Located in the Upper Triangle

	rs2550946	rs11564750	rs3776513	rs40184
rs2550946		0.0004	0.004	0.00002
rs11564750	0.16		0.0004	0.0002
rs3776513	0.0004	0.05		0.01
rs40184	0.02	0.002	0.08	

Linkage disequilibrium values determined by r^2 are present in the lower triangle.

TABLE IV. The Transmission of Alleles in a Four-Marker Haplotype

rs2550946	rs11564750	rs3776513	rs40184	T	NT	OR
A	C	G	C	43	54	0.80
A	C	G	T	26	29	0.90
A	C	T	C	0	9	0.00
A	C	T	T	5	12	0.42
A	G	G	C	142	137	1.04
A	G	G	T	112	119	0.94
A	G	T	C	2	4	0.50
A	G	T	T	56	54	1.04
G	C	G	C	1	3	0.33
G	C	G	T	0	1	0.00
G	C	T	C	0	1	0.00
G	G	G	C	279	177	1.58
G	G	G	T	122	149	0.82
G	G	T	C	28	32	0.88
G	G	T	T	48	83	0.58

Only one risk haplotype is observed highlighted in bold.

they had relatively high minor allele frequencies compared to the significantly associated SNPs.

Several other groups have also identified SNP associations with ADHD in the 5' region of *DAT1*. Genro et al. [2007] using a sample of ADHD probands from Brazil detected association to the same allele of rs2652511 reported in this study. Furthermore, they found that the effect size for this association was greater when they restricted their analysis to the subset of individuals with combined type ADHD. Ohadi et al. [2006] found association of ADHD with the T-allele of a core promoter SNP at position -67 (rs2975226) which is thought to possess functional properties. The T-allele of this SNP creates a putative SIF-binding element [Rubie et al., 2001] capable of increasing transcription by twofold [Greenwood et al., 2002; Greenwood and Kelsoe, 2003]. This putative functional SNP lies 4.8 kb downstream from (and is in high LD with) SNP rs2550946 that is associated with ADHD in this study; and only 774 bp from SNP rs2652511 that was associated with ADHD in the ST1 study [Brookes et al., 2006b]. This SNP also lies in a region of highly conserved sequence identity (63%) between the mouse and human 5' flanking regions [Donovan et al., 1995]. Finally, Friedel et al. [2007] recently completed a comprehensive analysis of 30 SNPs spanning the 3' and 5' regions of *DAT1* in a sample of ADHD cases from Germany. One of the associated markers identified in their study (rs403636) showed the same allelic association to that reported in our ST1 study but did not clearly identify an overlapping haplotype with that reported in this study.

The finding of replicated association with SNPs within the 5' regulatory region of *DAT1* is clearly of key interest and further work is needed to delineate the role of functional variation in this region. Drgon et al. [2006] reported that the rs2550946 is involved in altered transcriptional regulation, with the C-allele promoting Rps3a-2-homol-D transcription factor binding, whereas the T-allele promotes GHF1/Pit1-pr-1 binding. Based on the association with the C-allele in this study the association with ADHD might be mediated by increased binding of the transcription factor Rps3a-2-homol-D in ADHD cases. Drgon et al. [2006] also reported that the C-allele of SNP rs2652511, that was associated with ADHD in this study and is in strong LD with rs2550946, is associated with significantly higher levels of *DAT1* expression in vivo.

The second key finding from this study was the contribution of genetic variants to the risk for ADHD from both the 3' and 5' regions. However, it is not clear from the analysis of

association data alone what are the genetic sequences within these regions that are the primary source of the association signals. No single marker was responsible for the haplotype association and the evidence from WHAP indicated that the presence of genetic variation in both regions was important. The finding that only a single haplotype spanning the four SNP markers in this study was associated with increased risk for ADHD (odds ratio = 1.58, global $P = 0.00016$, haplotype specific $P = 1.7 \times 10^{-6}$) suggests a possible interaction between the two loci, but could also arise from a simple additive effect of two main associated loci. The possibility that risk variants at more than one site are required to enhance the risk for ADHD could contribute to the observed heterogeneity seen within meta-analytic studies of *DAT1* [Li et al., 2006].

The functional role of the two VNTR markers located within the 3'UTR and intron 8 in our earlier study also remains uncertain. In our previous report we found evidence for a contribution of both loci to a haplotype association between the two VNTRs [Asherson et al., 2007], however it was not possible to distinguish between two possible explanations. First that the two VNTR markers tag a single (unidentified) functional site; and second that there is a functional interaction between two VNTR markers (or closely associated functional variants). It is also feasible that the two VNTRs mark two independent loci with purely additive effects at the level of gene function. We have also analyzed the VNTR markers in relation to the SNPs investigated in this study and found that they formed part of the same 3' haplotype reported here (data not shown). We therefore conclude that analysis of *DAT1* in the IMAGE dataset suggests the presence of two and potentially three functional loci.

In summary we have replicated association findings between ADHD and genetic variants within *DAT1*, that were initially identified as nominal associations in a scan of SNP markers tagging genetic variation across 51 candidate genes. Limitations of this study include possible heterogeneity arising from the use of a multisite European sample, although this is largely controlled by the use of the Transmission Disequilibrium Test as our primary tool for association analysis. A further problem was the relatively small sample size of this study compared to the initial ST1 study, which might account for replication of only two out of the four markers studied. The overall findings in the combined IMAGE dataset suggest that there may be additive or interactional effects of two or more functional loci within *DAT1*. Further analysis of the functional consequences of genetic variation within the gene is therefore required and replication and meta-analytic studies in independent datasets are required to confirm or refute the pattern of findings reported here.

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